What is the optimal timing of thawing for transferring vitrified-warmed cleavage stage of slow-growing embryos? A cohort retrospective study

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Research

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Abstract

Background

To evaluate optimal thawing time, the early thawing or the routine thawing time, for transferring vitrified-warmed, and cultured overnight cleavage stage of the slow-growing embryos on Day 3 in frozen embryo transfer (FET) cycle.

Methods

This was a retrospective cohort study from January 2017 to July 2018, a total of 705 slow-growing embryos FET cycles in which the patients were aged < 40. Thawing cleavage stage slow-growing Day 3 embryos on either the 2nd or 3rd day after ovulation in natural cycle or the equivalent timing of the artificial cycles.

Results

For slow growing embryos, the clinical pregnancy rate of early thawing group (152/468 (32.5%)) was significantly higher than that of routine thawing group (55/235 (23.4%)) (OR 1.39(CI 1.06–1.81), p = 0.01), while there was no statistically significant difference in pregnancy loss in early thawing group (39/170 (22.9%)) versus in routine thawing group (16/62 (25.8%)) (OR 0.89 (CI 0.53–1.47), p = 0.65).

Conclusion

For slow-growing embryos, higher pregnancy outcomes were shown in early thawing strategy as compared to the routine thawing, which suggested that the improvement of endometrium-embryo synchronism may correct the time difference brought by the slow-growing embryos.

Background

Controlling the time overlap between embryo implantation and high receptivity of endometrium is the prerequisite for the success of embryo transfer [1]. Previous data showed that the implantation window phase last about 2 to 5 days [2], and higher pregnancy rate may be gained from the more paralleled development between embryos and endometrium[3, 4]. Recently, several studies emphasized the precise timing of thawing and transferring embryos in FET cycle. For instance, S. Tannus et al. pointed out that extending culture of Day 5 morula to Day 6 and subsequent FET could enhance the live birth rate, indicating that the implantation potential of Day 5 slow growing embryo could be rescued to some extent by improving the endometrium and embryo synchronization[5]. However, C. Blockeel et al. reported that there was no significant difference in the pregnancy outcome between the early and delayed transfer of vitrified-warmed cleavage stage embryo in FET cycles [6]. The contradictive results may be stemmed

from the different stages of embryos. Till now, information regarding the optimal thawing and transferring time especially for the slow-growing cleavage stage embryos is still lacking.

Generally speaking, blastomere number as 8 could predict the quality of Day 3 embryos with obtaining satisfied live birth rate [7]. However, not all embryos exhibit the same development speed, and study reported that slow-growing embryos may account for 30% of total [8]. Slow-growing Day 3 embryos refer to embryos which have 6 or fewer blastomeres [9], which some studies reported would have negative effects on embryo- endometrial synchrony and therefore decrease embryo implantation rate [10, 11]. Lewin A et al. showed that lower pregnancy rate gained from the implantation of slow-growing cleavage embryos compared to normal- growing embryos [12], while Heather Burks et al. indicated that adjusting the timing of a slow embryo transfer could lead to a comparable pregnancy outcome to that of a normal developing embryo [13]. It still lacks data that regulating the thawing and transferring time of slow-growing Day 3 embryos may improve clinical pregnancy outcome. Therefore, the goal of this analysis is to investigate the effect of thawing and implantation time of slow-growing embryos in cleavage phase on clinical outcome during FET cycle by thawing cleavage stage Day 3 embryos on either the 2nd or 3rd day after ovulation in natural cycle or the equivalent timing for the artificial cycles.

Materials And Methods

Study design

This was a retrospective cohort study initiated at the Center for Reproductive Medicine in the Peking University Shenzhen Hospital, China between January 2017 and July 2018. A total of 705 FET patients in which patients aged < 40 were included and 1486 embryos were formed, of which 1366 embryos were eventually transferred. All patients underwent only one FET cycle. In our research, the final transplanted slow- growing embryos were divided into early thawing group and routine thawing group according to the physician's decision, all of which were cultured overnight and were transferred one day later. This study has been approved by the Ethics Review Committee of Shenzhen Hospital of Peking University.

Ovarian stimulation, IVF or ICSI treatment, Embryo freezing and thawing

Embryos were gained from controlled ovarian stimulation with conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). All embryos were assessed according to the routine evaluation system [9] and were cryopreserved on Day 3 of embryo culture. All the cryopreservation was performed using vitrification protocols. For Day 3 vitrification, embryos with at least 4 blastomeres and ≤ 25% fragmentation were selected for cryopreservation. Only the slowing growing embryos which were composed of 4 to 6 blastomeres were included in this study.

Embryos were thawed at the following two different time points and classified into two different groups (Fig. 1). As for the natural cycle and controlled ovarian stimulation cycle, embryos were thawed after 2 days (early thawing group) or 3 days (routine thawing group) of ovulation. As for the hormone

replacement treatment (HRT) cycle, embryos were thawed after 3 days (early thawing group) or 4 days (routine thawing group) of progesterone administration.

Embryos were then warmed 1 day before transferring. The surviving embryos were cultured overnight and transferred at the 2nd day. Morphological survival was confirmed by counting the intact cells on the number of cells present at cryopreservation. Embryos with at least 50% intact cells were considered surviving and were further cultured. Further cleavage was evaluated the next morning and characterized into top quality, good quality and poor quality according to the previous standard [6]. Embryos with further cleavage and showing signs of compaction or blastulation were considered as top quality. Good quality embryos had at least eight blastomeres and further cleavage of at least two cells, while poor quality embryos had less than eight blastomeres and/or no further cleavage and/or limited further cleavage of only one cell. The further cleavage rate was defined as the percentage of embryos with at least division of two cells after one-day culture on the total number of transferred embryos.

Preparation of the endometrium

As for natural cycle and natural cycle with ovulation stimulation cycle: Ultrasound examination was applied to monitor the follicular growth from day 8–10 of the menstrual cycle and consecutively examined every 2–4 days. After the luteinizing hormone (LH) surge, dominant follicle collapse was confirmed as the ovulation day (day 0) [14]. Besides, ovulation day was double confirmed according to serum progesterone (P) level. If dominant follicle disappeared, P serum level was checked. In the natural cycle with hCG, after ultrasound evidence of follicles ≥ 18 mm and endometrium thickness ≥ 7 mm, 6,000 IU urinary hCG (Choriomon, IBSA, Lugano, Switzerland) was administered to trigger ovulation.

As for HRT cycle and GnRH-a pretreatment plus HRT cycle: before the start of the cycle, the basal hormone value of endocrine evaluation was confirmed by the measurement of estradiol (E2), P, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Then a dose of 2 mg estradiol valerate (Progynovaw, Bayer-Schering Pharma AG, Berlin, Germany confirm) twice a day was given to the patients for 7 days, followed by 6 days of estradiol valerate at a dose of 2 mg three times per day. On Day 13 of estradiol valerate treatment, endometrial thickness was measured by ultrasound and hormonal analysis was performed by serum E2 and P examination. In FET cycle, the same embryonic time convention is usually followed, and the date of ovulatory in the natural cycle is equivalent to the 1st day of P administration in the artificial cycle [15]. If the serum P level was higher than.1.5 ng/ml, the cycle was cancelled. If endometrial thickness was ≥ 7 mm, P supplementation was started. The choice for using GnRH-a was depended on the physician’s decision.

Assessment of pregnancy outcome

Conception was defined as arbitrary serum beta hCG is higher than 50 IU/L at 14 days following embryo transfer. Clinical pregnancy was confirmed by ultrasound scan with at least one fetus with heart beat by 7 weeks pregnancy. The implantation rate was that the number of gestational sacs observed divided by the number of embryos transferred and the live birth was defined as at least one baby born.

Outcome measures

The pregnancy outcome of FET cycle was studied, including conception, clinical pregnancy, implantation, biochemical miscarriage, first trimester pregnancy loss, second trimester pregnancy loss, ongoing pregnancy and livebirth.

Statistical analyses

The *p*-value, risk ratio and confidence interval were analyzed by Social Science 19 (SPSS, Inc., Chicago, IL, USA). The categorical data are expressed as percentage and analyzed by using the chi-square test or Fisher's accurate test according to the sample size. The odds ratio (OR) and 95% confidence interval (CI) were compared to evaluate the difference. According to the normality of the results, the continuous variables were analyzed by independent t test or Mann-Whitney u test, and all the data were two-tailed test. Logistic regression analysis was used to adjust for confounders, including regimen of endometrial preparation and the timing of thawing embryos. The significance level was set at $p < 0.05$.

Results

During the study period, 705 FET patients were initially included, of which 470 were in the early thawing group and 235 were in the routine thawing group. In the early thawing group, 2 patients were lost follow-up and 468 patients were included ultimately. In the routine thawing group, there was no lost follow-up patients, and 235 patients were included finally (Fig. 2). As indicated in Table I, there was no significant difference in the basic characteristics including age, height, weight, BMI, indication for IVF/ICSI and basic hormones between the early thawing group and the routine thawing group.

The cycle characteristics of the two groups was presented in Table II. There was no significant difference between the two groups in the choice of IVF or ICSI. The endometrial thickness on the transferring day was 11.3 ± 2.3 mm in early thawing group versus 11.2 ± 2.2 mm in routine thawing group. With an average of about 2 embryos per patient, there was no significant difference in the number of embryos transferred.

The embryonic characteristics was exhibited in Table III. The ratio of embryos transferred was 92.0% and 91.8% in the two groups, which showed no significant difference. There was no significant difference in the percentage of the further cleavage embryos between the two groups. After thawing and culturing overnight, the quality of embryos was scored and characterized as top quality, good quality and poor quality. There was no significant difference in the embryo quality distribution between the two groups.

As shown in Table IV, clinical outcomes, including conception per woman, clinical pregnancy rate, implantation rate, biochemical miscarriage rate, first trimester pregnancy loss, second trimester pregnancy loss, ongoing pregnancy and live birth (single and twin) rate were compared between two groups. The clinical pregnancy rate in the early thawing group (152/468(32.5%)) was significantly higher than that in the routine thawing group 55/235(23.4%)) (OR 1.39 (CI 1.06–1.81), $p = 0.013$), while there

was no statistically significant difference in pregnancy loss between the two groups (OR 0.89 (CI 0.53–1.47), p = 0.65). Besides, the implantation rate, ongoing pregnancy rate and live birth rate in the early thawing group were also significantly higher than that in the routine thawing group as depicted in the table IV.

The quality of embryos which obtained clinical pregnancy in the two groups was compared and the result demonstrated there was no significant difference in the percentage of top, good and poor quality between the two groups at the transfer day (Table V).

Additionally, logistic regression analysis of mixed factors with Clinical pregnancy were showed in table VI. Several variables, including regimen of endometrial preparation and the timing of thawing embryos, were employed in the logistic regression analysis to reduce the influences on clinical pregnancy outcomes. The results showed that the other three groups were shown to be associated with similar clinical pregnancy outcome compared with the natural cycle. Besides, the clinical pregnancy outcome of thawed embryos on day-2 was significantly higher than that on day-3.

Discussion

The synchronous development of embryo and endometrium is an important prerequisite for the success of embryo transfer. As for FET cycle, the development of endometrium and embryo are isolated [14]. Slow-growing embryos have lower implantation potential compared to normal embryos [10, 16]. When the embryos develop slowly, at the same time, elevated progesterone in vivo would lead to endometrium decidualization, and therefore a "time difference" would exist in the implantation of the embryos according to the routine time. The time difference between the slowing-growing embryos and the accelerated development endometrium may be main reasons why the implantation rate of the slow-growing embryo is lower than that of the normal embryo. For instance, M. W. Healy et al. compared the pregnancy outcome of implantation of slow-growing Day 5 and Day 6 embryos when premature progesterone promoted on the trigger day and found that lower live birth rate was shown on Day 6 embryos (45.6% versus 34.0%), indicating that endometrium-embryo synchronism decreases when slow-growing embryos encounter advanced endometrium over time [17]. In Kevin S. et al. study, the clinical pregnancy of blastocyst with similar implantation potential on the 5th, 6th and 7th day after ovulation was compared, lower clinical pregnancy rate was found on Day 7 blastocysts cryopreserved [18]. It seemed that the reason for low pregnancy rates of later developing blastocysts may origin from the asynchrony of embryos and endometrium instead of the slow development of the embryos. In our study, early thawing group obtained higher pregnancy outcome by transferred on the 2nd day after ovulation in natural cycle or the equivalent timing for the artificial cycles, just as the normally developed embryos are transferred after thawing on the 3rd day, which effectively resynchronizes the embryo to the development of the endometrium.

In addition, some studies extended the embryo culture to a certain degree of development, and compared them with the normal growing embryo of pregnancy outcomes. Heather Burks et al. analyzed the

implantation between those who reached 8 cells on Day 3 (normal embryo group) and those who gained 8 cells on Day 4 (delayed embryo group) and found similar pregnancy outcomes [13]. The above study suggested that the synchronization of endometrium and embryos may correct the time deviation brought by the slow development of the embryos, which had similar goal as this study. However, in that study, there was significant difference in infertile factors caused by ovarian reserve function between the delated Day 4 embryo group and the normal Day 3 embryo group (44.4% versus 16.4%, $p = 0.003$), which indicated that the limited comparability between the two groups. In our study, embryos with the same developmental starting point in two groups were thawed and transferred at different times respectively, which could strictly control variables.

In this research, the quality of embryos was evaluated after thawing and overnight-culture and characterized as top quality, good quality and poor quality. In order to exclude the effect of embryo differences on clinical pregnancy outcome, we evaluated the quality of embryos which gained clinical pregnancy and found that there were no significant differences between embryos with top quality, good quality or poor quality, which indicated that these pregnancy outcomes were not caused by embryonic factors. At the same time, our result exhibited that there was no significant difference in the miscarriage rate between two thawing strategies, while higher implantation rate was shown in early thawing group, which illustrated that the improvement of clinical pregnancy rate was achieved by increasing implantation rate instead of reducing miscarriage rate.

To the best of our knowledge, this is the first comparison of clinical outcomes between early thawing and routine thawing of day 3 slow-growing embryos. However, there are a few limitations in our study. Firstly, the methods of preparing endometrium were significantly different in the current study. A randomized controlled trial reported that similar outcomes shown in different types of endometrial preparation strategies for FET cycles, included natural cycle, natural cycle with ovulation stimulation cycle, HRT and GnRH-*a* pre-treatment plus HRT cycle [19], there is no current evidence that higher clinical outcomes can be obtained by using a certain method for the preparation endometrium of FET cycles [15, 20]. Besides, as showed on our multivariate regression analysis showed, endometrial preparation protocol inconsistency did not affect clinical pregnancy outcome. Secondly, single-center retrospective analysis limited the strength of the evidence of the current conclusion.

Conclusions

In conclusion, higher pregnancy outcomes obtained by early thawing of slow-growing embryos deserve clinicians' attention and is of guiding value for the determining of thawing and transferring programs. Higher quality randomized controlled studies are needed in the future to further confirm the role of early thawing and transfer of slow-growing embryos.

Abbreviations

ART: assisted reproductive techniques

IVF-ET: in vitro fertilization- embryo transfer

ICSI: intra-cytoplasmic spermatozoa injection

FET: frozen–thawed embryo transfer

LBR: live birth rate

AT: assisted hatching

D3: day 3

NC: natural cycle

HRT: hormone replacement therapy

P: progesterone

LH: luteinizing hormone

hCG: human chorionic gonadotropin

E2: estradiol

FSH: follicle-stimulating hormone

OR: odds ratio

CI: confidence interval

Declarations

Ethical Approval and Consent to participate

This study has been approved by the Ethics Review Committee of Shenzhen Hospital of Peking University.

Consent for publication

This manuscript is approved by all authors for publication.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that there is no competing interests.

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Authors' contributions

Lan Geng and Jia-hui Wu made major contributions to data analysis, article conception and writing. Luo Jia-qi and Shi Yu were responsible for data analysis. Zhen-hui Hou and Wei-ping Qian contributed to data collection and verification. Amanda Kallen and Xia Xi contributed to the research and design, critical discussion and review of manuscripts.

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Tables

Table I Demographic characteristics.

	Early thawing group N=468	Routine thawing group N=235
Age(years)[1]	32.0±3.8	32.3±4.0
Height(cm)[1]	159.1±4.8	159.0±4.4
Weight(kg)[1]	53.0±7.6	52.5±6.3
BMI (kg/m²)[1]	20.9±2.9	20.8±2.5
Indication for IVF/ICSI[2](n)		
Tubal factor	191(40.9%)	102(43.4%)
Ovulation factor	14(3.0%)	9(3.8%)
Endometriosis	25(5.3%)	8(3.4%)
Male factor	115(24.6%)	51(21.7%)
Combined factors	46(9.8%)	22(9.4%)
Unexplained infertility	52(11.1%)	30(12.8%)
Others	25(5.3%)	13(5.5%)
Hormone profile (Basal)		
FSH(IU/l)	8.0±2.9	8.3±3.2
LH (IU/l)	5.1±4.8	5.1±2.4
E2[3](ng/l)	55.2±38.4	54.8±38.8
P (μg/l)	0.7±0.5	0.7±0.4
AMH[4] (ng/ml)	5.0±4.1	5.2±4.5

[1]Data are expressed as mean values+ standard deviation (SD).

[2]Data are expressed as absolute values (percentage).

[3]E2 was missing for 27 patients in the early thawing group and for 10 patients in the routine thawing group.

[4]AMH was missing for 5 patients in the early thawing group and for 1 patient in the routine thawing group.

Table II Cycle characteristics.

	Early thawing group N=468	Routine thawing group N=235
IVF (n)	281(60.0%)	144(61.3%)
ICSI (n)	161(34.4%)	80(34.0%)
Half ICSI (n)	26(5.6%)	11(4.7%)
Regimen of endometrial preparation (n)		
Natural cycles	149(31.8%)	41(17.4%)
Hormone replacement treatment (HRT)	182(38.9%)	136(57.9%)
Controlled ovarian stimulation cycle	106(22.7%)	32(13.6%)
GnRH-a pretreatment plus HRT cycle	31(6.6%)	26(11.1%)
Endometrial thickness at embryo transfer day (mm)[1]	11.3±2.3	11.2±2.2
Embryos transferred[1]	2.0±0.4	1.9±0.3
Cycles with at least 1 top quality embryo/embryo transfer[2]	64/132(48.5%)	28/55(50.9%)

[1]Data are expressed as mean values+ standard deviation (SD).

[2] The data is shown as the ratio of the mentioned value to the total number of samples (percentages between brackets).

Table III Embryonic characteristics.

	Early thawing group N=468	Routine thawing group N=235	P-value
n thawed	996	490	
n embryos surviving (%)	925(92.9%)	464(94.7%)	NS
n transferred (%)	916(92.0%)	450(91.8%)	NS
Further cleavages (%/transferred)	636(68.8%)	309(66.6%)	NS
n top quality	76(8.2%)	32(6.9%)	NS
n good quality	205(22.2%)	110(23.7%)	NS
n poor quality	644(69.6%)	322(69.4%)	NS

Table IV Outcome measures.

	Early thawing group N=468	Routine thawing group N=235	OR (95%CI)	P-value
Pregnancy				
Conception per woman	170(36.3%)	62(26.4%)	1.38(1.08-1.76)	0.01
Clinical pregnancy per woman	152/468(32.5%)	55/235(23.4%)	1.39(1.06-1.81)	0.01
Implantation per embryo	182/916(19.9%)	69/450(15.3%)	1.30(1.01-1.67)	0.04
Pregnancy loss	39/170(22.9%)	16/62(25.8%)	0.89(0.53-1.47)	0.65
Biochemical miscarriage	17/170(10%)	7/62(11.3%)	0.89(0.39-2.03)	0.78
First trimester pregnancy loss	19/152(12.5%)	7/55(12.7%)	0.98(0.44-2.21)	0.97
Second trimester pregnancy loss	3/152(2.0%)	2/55(3.6%)	0.54(0.09-3.16)	0.61
ectopic pregnancy	1/170	1/62		
Ongoing pregnancy per woman	133/468(28.4%)	47/235(20.0%)	1.42(1.06-1.91)	0.02
Livebirth (total)	130/468(27.8%)	45/235(19.1%)	1.45(1.07-1.96)	0.01
Singleton livebirth per woman	110/468(23.5%)	37/235(15.7%)	1.49(1.07-2.09)	0.02
Twin livebirth per woman	20/468(4.3%)	8/235(3.4%)	1.26(0.56-2.81)	0.58

Data are presented as number of cases including nominator and denominator values (percentages between brackets).

Table V The quality of embryos which obtained clinical pregnancy at the transfer day

	Early thawing group N=152	Routine thawing group N=55	P-value
n transferred	304	112	
n top embryo	31(10.2%)	10(8.9%)	NS
n good embryo	90(29.6%)	37(33%)	NS
n poor embryo	183(60.2%)	65(58.1%)	NS

Table VI. Multivariable analyses of Mixed factors with Clinical pregnancy.

Characteristic	OR	95%CI	P
Regimen of endometrial preparation			
Natural cycles vs. HRT	0.92	0.59-1.42	0.71
Natural cycles vs. COH	1.17	0.70-1.94	0.54
Natural cycles vs. GnRH-a + HRT	1.39	0.71-2.71	0.33
Thawing time			
Day-2/ Day-3	0.63	0.43-0.94	0.01

Figures

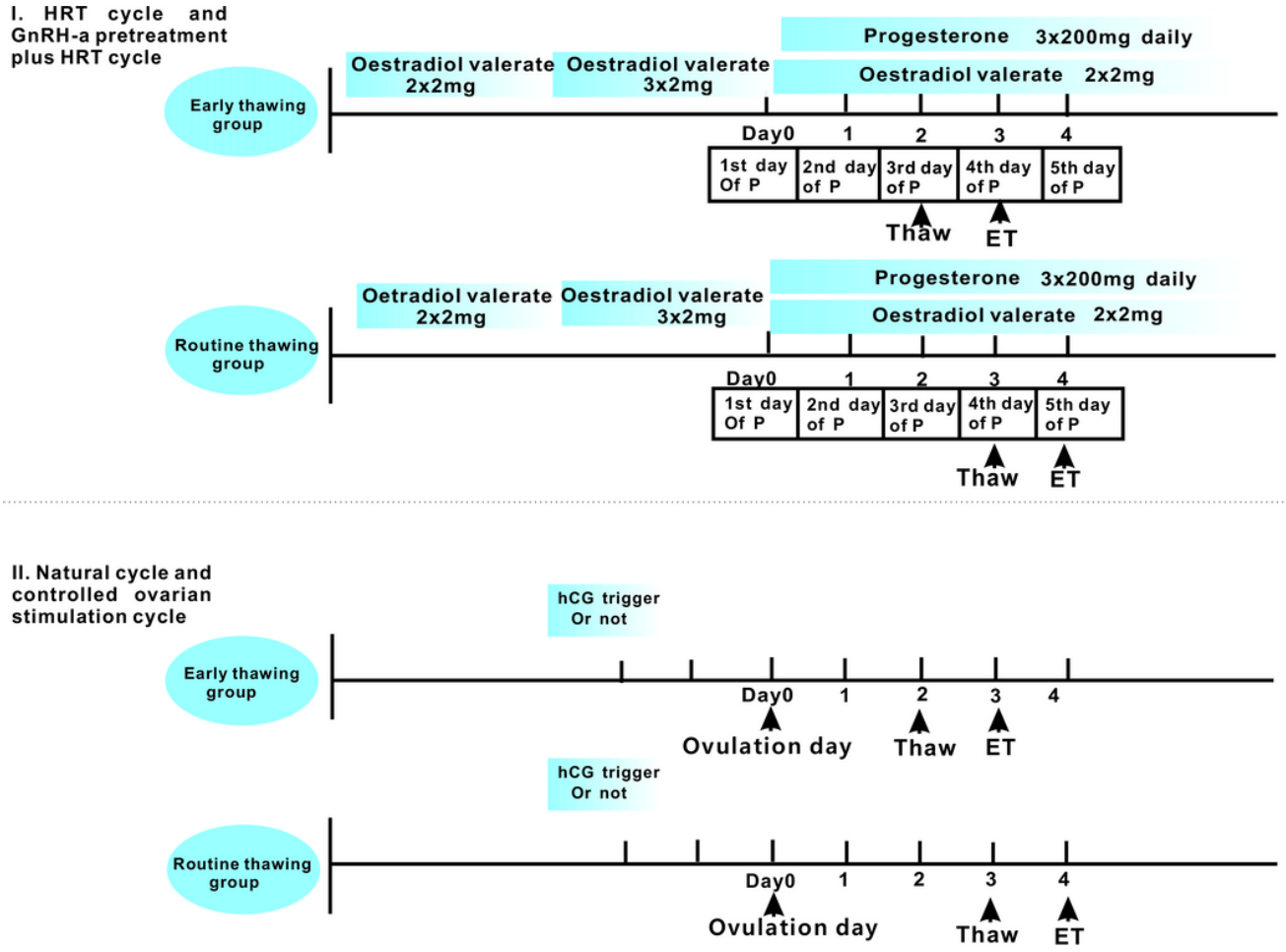

Figure 1

Embryos were thawed at the following two different time points and classified into two different groups (Figure 1).

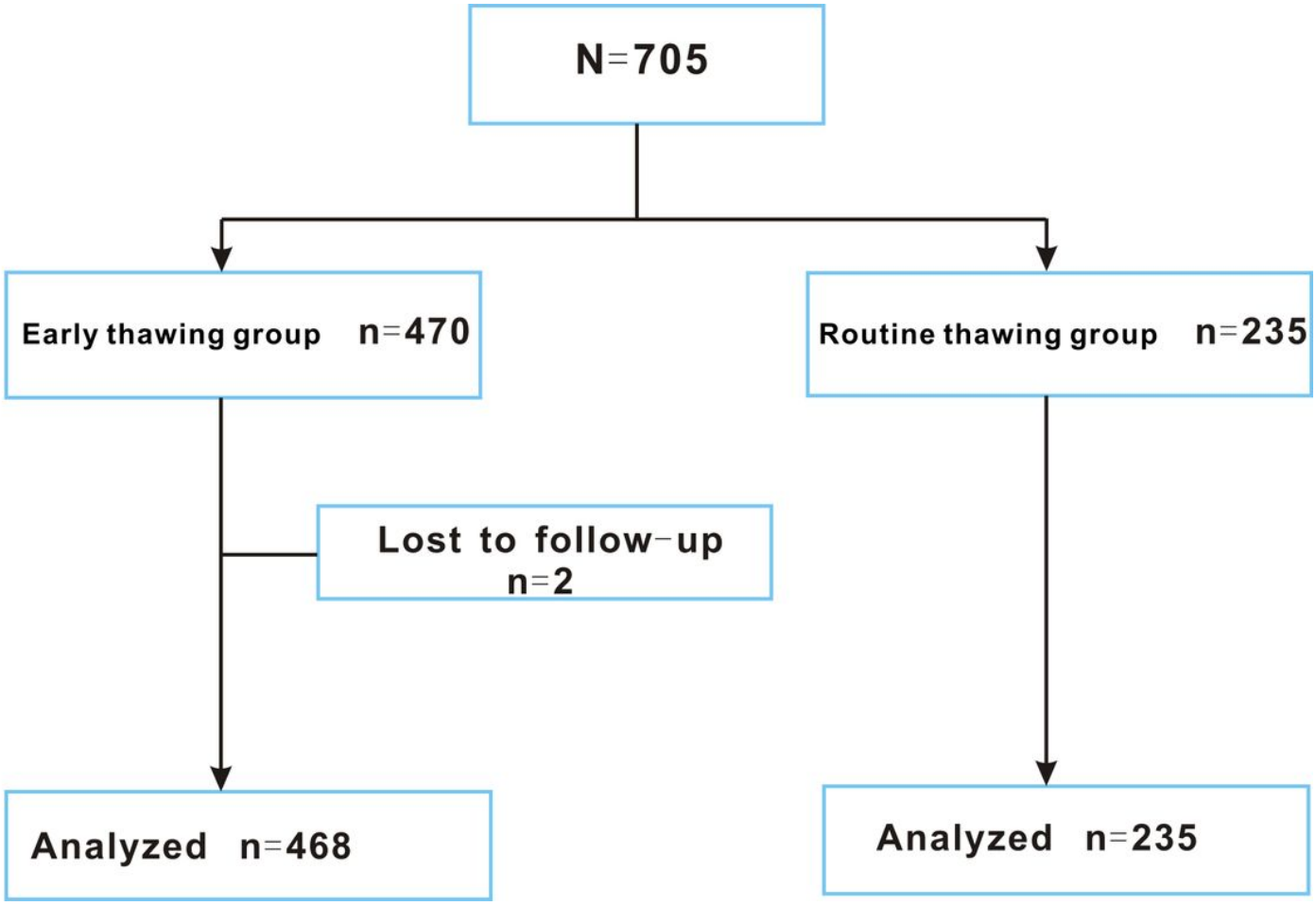

Figure 2

In the routine thawing group, there was no lost follow-up patients, and 235 patients were included finally (Figure 2).